Huynh, Phuong N.
Tuesday, January 30, 2001 6:00 PM
STIC-ILL
RE: 09/270,910 Rush

protein exp. purification

Hi!

Please deliver the following references:

1. Prot Exp Purification 8: 365-373 (1996).

2. Clin. Exp. Allergy 22: 391-9 (1992)

3. Nature structural biology 3: 1040-45 (1996).

Thanks,
Neon
Art unit 1644
Mail CM1 9E12
Tel 308-4844

PROTEIN EXPRESSION AND PURIFICATION 8, 365–373 (1996)
ARTICLE NO. 0112

Characterization of Purified Recombinant Bet v 1 with Authentic N-Terminus, Cloned in Fusion with Maltose-Binding Protein

Michael D. Spangfort,[1] Henrik Ipsen, Susanne H. Sparholt, Stig Aasmul-Olsen, Martin R. Larsen,* Ejvind Mørtz,* Peter Roepstorff,* and Jørgen N. Larsen

Research Department, ALK-ABELLÓ Group, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark; and
**Department of Molecular Biology, Odense University, DK-5230 Odense, Denmark*

Received March 15, 1996, and in revised form July 12, 1996

A gene encoding the pollen major allergen Bet v 1 from *Betula verrucosa* (White Birch) has been cloned and expressed in *Escherichia coli* as a fusion with maltose-binding protein and a Factor Xa proteolytic cleavage site. A generally applicable cloning strategy based on polymerase chain reaction was designed to position the Factor Xa proteolytic site so that the authentic amino terminus of Bet v 1 was generated after cleavage. Fusion protein was isolated by amylose affinity chromatography and enzymatically cleaved by incubation with Factor Xa. Recombinant Bet v 1 was isolated by gel filtration and gave rise to a single band with apparent molecular weight of 17 kDa when analyzed by SDS–polyacrylamide gel electrophoresis. N-terminal sequencing of the first 20 amino acids showed complete agreement with the deduced Bet v 1 DNA sequence. Mass spectrometry showed that recombinant Bet v 1 has a molecular mass of 17,440 ± 2 Da; 86% of the recombinant Bet v 1 amino acid sequence could be verified by digestion with Lys-C and mass spectrometric peptide mapping. The yield of purified recombinant Bet v 1 was 10 mg per liter *E. coli* cell culture. Two-dimensional gel electrophoresis of purified recombinant protein gave rise to one major protein spot and one or two minor spots focusing at slightly different pHs. The immunochemical properties of recombinant protein were indistinguishable from those of naturally occurring Bet v 1 when compared using a panel of murine monoclonal antibodies and serum IgE from birch pollen allergic patients. Furthermore, recombinant Bet v 1 elicited T-cell proliferation comparable to that of natural Bet v 1. Thus, the methods used for bacterial expression and protein purification result in relatively high yields of folded recombinant Bet v 1 with correct N-terminal sequence and molecular mass. Furthermore, the B- and T-cell epitope structures of recombinant Bet v 1 closely resemble those of the natural protein from pollen. © 1996 Academic Press, Inc.

Tree pollens are a major source of airborne allergens causing IgE-mediated (Type I) allergic symptoms during early spring (1). The major allergen of Birch (*Betula verrucosa*) pollen has been identified as a 17-kDa protein denoted Bet v 1 (2–4). More than 95% of birch pollen allergic patient's serum IgE reacts with Bet v 1 when analyzed by crossed radioimmunoelectrophoresis (5). Birch pollen allergic patient's IgE show cross-reactivity between Bet v 1 and the major pollen allergens Aln g 1, Car b 1, and Cor a 1 from other tree species of *Fagales* (6,7). This indicates similarities in B-cell epitope structures in agreement with the approximately 75% amino acid identity between the major allergens (8–11). A number of T-cell epitopes, evenly distributed along the primary amino acid sequence of Bet v 1, have been mapped using overlapping synthetic peptides (12). The biological function of Bet v 1 is still not fully clarified but it shows a high sequence homology to a family of pathogenesis-related proteins (8,13). Furthermore, based on *in vitro* biological activity, it has recently been suggested that Bet v 1 is a pollen-specific RNase (14). When analyzed by 2-D gel electrophoresis and silver staining, birch pollen extract shows a large number of protein spots representing different proteins in various isoallergens. About 20 of these protein spots can be identified as Bet v 1 isoallergens by use of monospecific antibodies raised against purified naturally occurring Bet v 1 (15,16). Analysis of IgE

[1] To whom correspondence should be addressed. Fax: (45) 45 76 16 82. E-mail: Michaels@inet.uni-c.dk.

1046-5928/96 $18.00

from individual birch pollen allergic patient's sera shows differences in reactivity toward different Bet v 1 protein spots, indicating that the isoallergens differ in epitope structure and amino acid composition (17,18). In addition, several different Bet v 1 cDNA sequences have been described (19). However, a detailed characterization of the epitope structure and molecular properties of Bet v 1 is hindered due to difficulties of isolating individual Bet v 1 isoallergens from pollen extract. This can to some extent be overcome by using molecular biological methods to produce single Bet v 1 isoallergens as recombinant proteins. Furthermore, an important issue is to what extent the observed heterogeneity of Bet v 1 is a nonphysiological artifact generated by the 2-D electrophoresis system. This can be addressed by electrophoretic analysis of individual purified recombinant isoallergens.

Cloning and bacterial expression of tree and grass pollen allergens have previously been performed in *Escherichia coli* either directly (20,21) or as fusion proteins (22–24). A major pollen allergen of rye grass, Lol p II (22), was expressed and characterized in the form of a fusion protein only, whereas recombinant allergens from olive tree pollen, Ole e I (23), and ragweed, Amb a II (24), contained up to 10 nonrelevant amino acid residues after purification. Expression of Bet v 1 in *E. coli* without a fusion partner resulted in low yields (20,21). Thus, there is a need for a bacterial or eucaryotic expression system which gives high yields of soluble recombinant allergens with the correct amino acid composition and sequence. This is of special importance if recombinant allergens are to be used as therapeutic agents.

In this work, we have expressed Bet v 1 fused to maltose-binding protein which has allowed purification of relatively large amounts of homogeneous recombinant Bet v 1 by a simple three-step isolation procedure. In addition, a generally applicable cloning strategy based on PCR[2] was used to ensure that correct N-terminal amino acids of recombinant protein were obtained after Factor Xa treatment. Purified recombinant protein has been characterized by a combination of immunological and protein chemical methods and compared to naturally occurring Bet v 1.

MATERIALS AND METHODS

Pollen extract and isolation of natural Bet v 1. Preparation of pollen extract from *B. verrucosa* (Allergon, Sweden) and isolation of naturally occurring Bet v 1 were performed as in Ref. (6).

Pollen allergic patients' sera. Tree pollen allergic patients' serum IgE pool was a mixture of equal volumes of serum from 11 donors. Patients were selected on the basis of case history, nasal or bronchial provocation tests, skin prick tests, and RAST, as described (25).

Antibodies against Bet v 1. Rabbit antibodies raised against purified Bet v 1 were prepared as described (6). Monoclonal hybridoma antibodies were derived from Balb/c mice immunized with purified tree pollen allergen. All monoclonal antibodies were of the IgG1 isotype, and supernatants from cells grown in the absence of serum were used.

mRNA preparation from pollen. RNA was purified from *B. verrucosa* pollen by phenol extraction and LiCl precipitation, as described (9). Oligo(dT)-cellulos (Boehringer Mannheim) affinity chromatography was performed batchwise in Eppendorf tubes.

Gene cloning. Molecular biological methods not described in detail below were performed according to standard procedures (26,27). Double-stranded cDNA was synthesized using a commercially available kit (Amersham). The gene encoding *Bet v 1* was specifically amplified by PCR and cloned in pKK233-2 (28). In brief, the primers, which were designed to match the sequence of the cDNA in positions corresponding to the amino terminus of Bet v 1 purified from birch pollen extract and the 3′-untranslated region, respectively, contained restriction sites *Nco*I and *Hind*III for directional cloning.

Subcloning. The gene encoding Bet v 1 was subsequently subcloned into the maltose-binding protein fusion vector pMAL-c (New England Biolabs). A DNA fragment was obtained by PCR using the following primer sequences.

Primer No. 1: 5′- C GCG GTA CCC ATC GAG GGT CGC GGT GTN TTY AAY TAY GAR AC
Primer No. 2: 5′- GGG GTA CCG AAT TCA TTA GTT GTA GGC ATC

The promoter proximal primer No. 1 is a degenerate 42-mer oligonucleotide containing a *Kpn*I site (underlined), four codons representing a Factor Xa cleavage site (italicized), and 20 nucleotides representing the amino-terminal sequence of Bet v 1, taking into account the degeneracy of the genetic code. The promoter distal primer No. 2 is designed to match the sequence surrounding the stop codon (italicized), and also contains a *Kpn*I site for cloning into pMAL-c. PCR was performed in a volume of 0.1 ml containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each dNTP, 0.1 μM of each primer, 0.01% (w/v) gelatin,

[2] Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; SBTI, soy bean trypsin inhibitor; DTT, dithiothreitol; LB medium, Luria–Bertani medium; PCR, polymerase chain reaction; PPD, purified protein derivative; RAST, radioallergosorbent test.

approx 2 μg template DNA, and 25 U/ml AmpliTAQ polymerase (Cetus/Perkin–Elmer). DNA polymerase was added after an initial 5-min incubation at 95°C. Template DNA was half of a plasmid DNA minipreparation. Twenty temperature cycles of 1 min at 94°C, 2 min at 42°C, and 3 min at 72°C were performed in a Perkin–Elmer/Cetus DNA thermal cycler. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. A band corresponding to the expected size of 517 base pairs was excised from the gel and electroeluted in an analytical unidirectional electroelutor (IBI). The DNA fragment was cut with *Kpn*I and ligated to pMAL-c cut with the same enzyme and treated to minimize selfligation with alkaline phosphatase. Ligation was allowed to proceed for 2 h at room temperature and the ligation mixture was used for transformation of *Escherichia coli* K-12 strain DH5α (29).

Nucleotide sequencing. Plasmid DNA was prepared for sequencing by purification using a commercially available kit (Qiagen). Nucleotide sequencing was performed using the dideoxy-nucleotide chain termination method (30) with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corporation).

Expression and purification of maltose-binding protein Bet v 1 fusion protein. For purification of fusion protein, recombinant *E. coli* cells were grown in 1 liter LB medium at 37°C to an optical density of 0.8 at 436 nm. Expression of fusion protein was induced by addition of 100 mM IPTG. Cells were harvested 3 h after IPTG induction by centrifugation (4000*g,* 20 min) and resuspended in 50 ml of 10 mM $MgCl_2$, 1 mM EDTA, 3 mM PMSF, 1 mM SBTI, 1 mM benzamidine, 5 mM DTT, 50 mM Tris-HCl, pH 7.5. The yield from 1 liter cell culture was about 6 grams of wet weight cells. Cells were broken by ultrasonic treatment with a Branson sonicator (Model 250, 6 min, 50% duty cycle at setting 7) whereafter cell debris and insoluble material were removed by centrifugation 8000*g* for 2 h at 4°C. Cell lysate supernatant was incubated 1 hr with 1 mg/ml DNase (Boehringer Mannheim) at room temperature and then extensively dialyzed against 200 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine, 20 mM Tris-HCl, pH 7.4, before amylose affinity chromatography.

Affinity chromatography of fusion protein. Escherichia coli cell lysate containing expressed fusion protein was loaded onto a 50-ml column of amylose resin (BioLabs, New England) equilibrated with 200 mM NaCl, 1 mM EDTA, 5 mM DTT, 20 mM Tris-HCl, pH 7.4. Bound fusion protein was eluted by 10 mM maltose dissolved in the same buffer and dialyzed against 20 mM Tris-HCl, pH 7.8, for 16 h at 4°C.

Enzymatic cleavage of fusion protein. Affinity-purified fusion protein was cleaved into its two protein constituents by incubation with Factor Xa (BioLabs, New England), at a fusion protein to Factor Xa weight ratio of 350:1, for 48 h at room temperature in 100 mM NaCl, 2 mM $CaCl_2$, 0.01% (w/v) SDS, 20 mM Tris-HCl, pH 8.0.

Isolation of recombinant Bet v 1. After enzymatic cleavage of fusion protein, recombinant Bet v 1 was isolated by FPLC molecular sieve chromatography using Sephadex G-75 HiLoad 16/60 (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0. Fractions containing recombinant Bet v 1 were pooled and concentrated using an Amicon pressure cell equipped with a 10-kDa cutoff filter. When necessary, residual amounts of maltose-binding protein were removed from the final recombinant Bet v 1 preparation by another round of amylose affinity chromatography and dialysis as described above. Purified recombinant Bet v 1 was stored at 4°C and was stable for several months.

T-cell proliferation assays. A Bet v 1 reactive T-cell line was generated from a birch pollen allergic patient by standard techniques as described (31). Antigen-specific T-cell proliferation was assessed by stimulation of T cells, 10^4/well, by serial dilution of antigen using irradiated (5000 rad) autologous Epstein Barr virus-transformed B cells as antigen-presenting cells in complete RPMI 1640 medium (Gibco) containing 5% heat inactivated, human AB serum. The cells were cultured for 4 days in a humidified atmosphere of 5% CO_2, 37°C, the last 15–18 h in the presence of tritiated methyl thymidine, 0.5 μCi/well (Du Pont, Germany). The cultures were harvested onto glass fiber filters and specific thymidine incorporation was assessed as mean counts per minute (cpm) of triplicate cultures. Stimulation indices values were calculated as the ratio of cpm values between stimulated and unstimulated cultures.

Electrophoresis and immunoelectrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to Ref. (32) using 16% polyacrylamide gels. Molecular weight markers (Bio-Rad Laboratories) used were phosphorylase b (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (42,699 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da). Two-dimensional isoelectric focusing/SDS–polyacrylamide gel electrophoresis was performed as in Ref. (17) using Immobiline gels (Pharmacia), pH range 4–7, for separation in the first dimension, followed by SDS–polyacrylamide gel electrophoresis. Crossed immunoelectrophoresis and immunoblotting were performed as in Ref. (6,32).

N-terminal sequencing. For N-terminal sequencing, purified recombinant Bet v 1 was electroblotted to PVDF membrane from 16% SDS–polyacrylamide gels and sequenced as in Ref. (33).

Mass spectrometry. Mass spectrometric analysis was performed with a Vestec Model 201 single quadrupole electrospray mass spectrometer (Vestec Corpora-

tion, Houston, TX). The sample containing recombinant Bet v 1 was desalted by microbore HPLC using a Nucleosil C_4 column. Prior to analysis by electrospray mass spectrometry, Bet v 1 was lyophilized and dissolved in a drop of 70% formic acid and diluted to a final concentration of 5 pmol μl^{-1} with 1% acetic acid: 50% methanol: 49% ultra-high-quality water. The sample was introduced into the mass spectrometer via a syringe pump with a flow rate of 0.3 μl min^{-1}. The spray voltage was 2.4 kV. The nozzle voltage was 200 V, the repeller voltage was set at 13 V, and the source block temperature was set at 150°C. Spectra were recorded with a scan rate of 10 s per scan with a mass window of *m/z* 600–1800. Mass calibration was performed using recombinant bovine acyl-CoA-binding protein.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) was performed on a Bruker reflex time-of-flight instrument using α-cyano-4-hydroxy cinnamic acid (Sigma) as matrix. Peptide mixtures of recombinant Bet v 1 digested with Lys-C (Promega) and Asp-N (Boehringer Mannheim) were diluted to 1 pmol/μl and 0.5 μl deposited on the matrix surface. Spectra were obtained by averaging 100–200 single-shot spectra and calibrated using matrix peaks as internal calibrants. The measured masses were used to identify the corresponding peptides in the Bet v 1 sequence.

RESULTS

Construction of expression vector. In this study, the gene encoding Bet v 1 was subcloned into the protein fusion expression vector pMAL-c. In order to generate the authentic amino terminus of Bet v 1 after Factor Xa cleavage, PCR was applied to position the Factor Xa protease cleavage site immediately in front of the amino-terminal glycine of Bet v 1. To accomplish this, the four codons representing the Factor Xa recognition sequence need to be included in the promoter proximal PCR primer, which becomes relatively long and therefore likely to induce primer–dimer formation and mispriming. For these reasons only 20 cycles were performed and in addition a high annealing temperature and a low primer concentration were chosen. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. One heavy band of the expected molecular size was noted. Additional bands were present in the high-molecular-weight range corresponding to the size of the plasmid used as template whereas no primer–dimer artifact was detected (results not shown). Nucleotide sequencing of the resulting construction confirmed that the sequence of the Bet v 1 encoding gene was unchanged by the subcloning procedure. The nucleotide and deduced amino acid sequences of recombinant Bet v 1 are shown in Fig. 1.

Purification of recombinant Bet v 1. Escherichia coli K-12 strain DH5α cells containing the recombinant plasmid were grown in LB medium and harvested 4 h after IPTG induction. Soluble lysate was analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 2). In contrast to noninduced cells, the IPTG-treated cells showed the presence of an additional protein band with an apparent molecular weight of about 60 kDa. The molecular weight is consistent with the molecular mass of Bet v 1 in fusion with the 43-kDa maltose-binding protein. Furthermore, immunoblotting using monospecific polyclonal antibodies raised against naturally occurring Bet v 1 showed positive reaction only to the 60-kDa protein band (not shown). No reaction was detectable against noninduced cells. These observations suggest that the fusion construct is expressed and contains recombinant Bet v 1 with preserved antigenic activity. Rocket immunoelectrophoresis using polyclonal antibodies against Bet v 1 showed that most of the expressed fusion protein was recovered in the recombinant cell lysate supernatant, whereas approximately 30% was found in the insoluble cell debris fraction.

The 60-kDa fusion protein bound to an amylose resin column with high affinity and could be efficiently isolated directly from recombinant *E. coli* cell lysate by a single affinity–chromatography step. Recombinant cells were broken by sonication and loaded onto an amylose resin column after removal of cell debris by centrifugation. After washing, the fusion protein was recovered from the column in a single distinct fraction by elution with 10 mM maltose and showed a high degree of purity as judged by silver-stained SDS–polyacrylamide gel electrophoresis (Fig. 2).

The purified fusion construct was thereafter enzymatically cleaved into its two protein constituents by incubation with Factor Xa. In order to optimize the proteolytic cleavage, different additives were tested. Addition of 5–10 mM DTT or 4 M urea inhibited the reaction whereas 2 M urea, 10 mM maltose, or 0.5% (w/v) of the nonionic detergent octyl-β-D-glucopyranoside only had marginal positive effect. The best result was obtained by addition of 0.01–0.1% (w/v) SDS and 0.1–2 mM $CaCl_2$ which resulted in almost complete cleavage of the fusion construct (Fig. 2). The enzymatic treatment yielded two proteins with apparent molecular weights of 40 and 17 kDa (Fig. 2). These were separated by gel filtration and the 17-kDa protein was identified as Bet v 1 by N-terminal sequencing and immunochemical assays. N-terminal sequencing verified that the first 20 amino acid residues were identical to the sequence deduced from the DNA sequence (Fig. 1). Isolated recombinant Bet v 1 comigrated with naturally occurring Bet v 1 when subjected to SDS–polyacrylamide gel electrophoresis, indicating similar apparent molecular weights. The molecular mass of recombinant Bet v 1 was further determined by electrospray mass spectrometry to be 17,440 ± 2 Da, which is in agree-

GGT GTT TTC AAT TAC GAA ACT GAG ACC ACC TCT GTT ATC CCA GCG

G V F N Y E T E T T S V I P A

GCT CGA CTG TTC AAG GCC TTT ATC CTT GAT GGC GAT AAT CTC TTT

A R L F K A F I L D G D N L F

CCA AAG GTT GCA CCC CAA GCC ATT AGC AGT GTT GAA AAC ATT GAA

P K V A P Q A I S S V E N I E

GGA AAT GGA GGG CCT GGA ACC ATT AAG AAG ATC AGC TTT CCC GAA

G N G G P G T I K K I S F P E

GGC TTC CCT TTC AAG TAC GTG AAG GAC AGA GTT GAT GAG GTG GAC

G F P F K Y V K D R V D E V D

CAC ACA AAC TTC AAA TAC AAT TAC AGC GTG ATC GAG GGC GGT CCC

H T N F K Y N Y S V I E G G P

ATA GGC GAC ACA TTG GAG AAG ATC TCC AAC GAG ATA AAG ATA GTG

I G D T L E K I S N E I K I V

GCA ACC CCT GAT GGA GGA TCC ATC TTG AAG ATC AGC AAC AAG TAC

A T P D G G S I L K I S N K Y

CAC ACC AAA GGT GAC CAT GAG GTG AAG GCA GAG CAG GTT AAG GCA

H T K G D H E V K A E Q V K A

AGT AAA GAA ATG GGC GAG ACA CTT TTG AGG GCC GTT GAG AGC TAC

S K E M G E T L L R A V E S Y

CTC TTG GCA CAC TCC GAT GCC TAC AAC

L L A H S D A Y N

FIG. 1. Nucleotide and deduced amino acid sequences of recombinant Bet v 1. Identities of amino acids verified by N-terminal sequencing are in bold. Peptides identified by MALDI-MS after enzymatic cleavage of recombinant Bet v 1 are underlined.

ment with the mass calculated from the cDNA sequence of 17439.7 Da. The primary sequence of recombinant Bet v 1 was verified by mass spectrometric peptide mapping after digestion with Lys-C and Asp-N. The Lys-C peptides cover 86% of the Bet v 1 sequence; only the C-terminal peptide is missing, as illustrated in Fig. 1. However, the C-terminal peptide was found after digestion with Asp-N (not shown). The mass spectrometric analysis of the intact recombinant protein and its peptides showed that the final protein preparation was homogeneous with respect to molecular mass and, since it is in accordance with that calculated from the Bet v 1 cDNA sequence, no posttranslational modifications seem to be present. The final yield of purified nonfusion recombinant Bet v 1 was about 10 mg per liter *E. coli* cell culture (see Table 1).

2-D gel electrophoresis of recombinant Bet v 1. The isolated recombinant Bet v 1 was further characterized by 2-D electrophoresis (Fig. 3), where it gave rise to one major spot focusing closely to its theoretical isoelectric point of 5.37. In addition, one or two less intense spots were also detected with same apparent molecular weight but focusing at slightly different pH values (estimated pH values 5.71 and 5.14, respectively). As no heterogeneity of the recombinant Bet v 1 protein could be detected by mass spectrometry, the observed charge heterogeneity may represent an artifact generated during the 2-D gel electrophoresis (for example, carbamylation, deamination, or alkylation of ϵ-amino groups in Bet v 1 lysine residues). When recombinant Bet v 1 was mixed with birch extract and analyzed on 2-D gels, no new protein spots

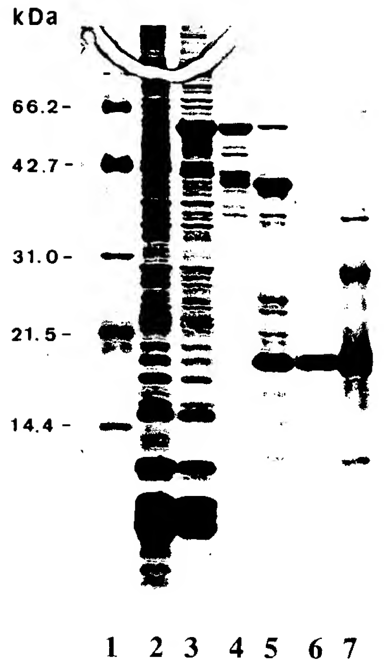

FIG. 2. Silver-stained SDS–polyacrylamide gel of different fractions from the purification of recombinant Bet v 1. Molecular weight markers (lane 1), recombinant *E. coli* cells (lane 2), recombinant *E. coli* cells 4 h post-IPTG induction (lane 3), fusion protein isolated by amylose-affinity chromatography (lane 4), fusion protein after incubation 48 h with Factor Xa (lane 5), purified recombinant Bet v 1 (lane 6), natural Bet v 1 purified from birch pollen extract (lane 7).

could be detected. However, the silver-staining intensity of two or three spots already present in pollen extract showed a significant increase.

Immunological characterization. The presence of conformational epitopes and the antigenicity of recombinant Bet v 1 was tested using a panel of murine monoclonal and rabbit polyclonal antibodies (see Fig. 4). Monoclonal antibodies raised against naturally occurring Bet v 1 reacted strongly with recombinant protein in immunoblotting and, in addition, both recombinant protein and natural Bet v 1 (20) cross-reacted with a monoclonal antibody raised against the major hornbeam pollen allergen Car b 1. Polyclonal antibodies of rabbit origin raised against purified birch pollen Bet v 1 or against birch pollen extract also showed strong positive reaction with recombinant Bet v 1. No reaction was seen when recombinant protein was tested against an antibody raised against a major allergen from *Chladosporium herbarum*.

Figure 5 shows an immunoblot of birch pollen extract when tested against a serum pool derived from birch pollen allergic patients. About 90% of the detected IgE-binding occurs against Bet v 1, confirming it as the major IgE-binding protein in birch pollen extract. Purified recombinant Bet v 1 also strongly bound birch pollen allergic patients serum IgE on immunoblots, demonstrating that natural and recombinant protein have comparable antigenic properties.

In order to test the presence of T-cell epitopes, recombinant Bet v 1 was further characterized by its ability to induce T-cell proliferation in a long-term Bet v 1-reactive T-cell line (Table 2). T cells derived from a birch pollen allergic patient were stimulated with 10 μg/ml of Bet v 1 purified from birch pollen or with recombinant Bet v 1 at the same concentration. In both cases a strong T-cell proliferation response occurred as judged from [^{3}H]thymidine incorporation. The stimulation indices for recombinant and natural Bet v 1 were 61.3 and 93.5, respectively.

DISCUSSION

Protein fusion constructs for heterologous expression in *E. coli* are widely used and often result in high yields of expressed soluble recombinant fusion protein. However, a major disadvantage is that the fusion construct often cannot be enzymatically cleaved to yield a recombinant protein with the authentic N-terminal amino acid. In many cases, the cleavage reaction results in a recombinant protein with one or more extra amino acid residues at the N-terminal. This might not always influence protein folding or enzymatic activity; however, it can be of major importance with respect to the immunochemical properties of the recombinant protein. In this study, we have used a novel PCR-based strategy to position the Factor Xa cleavage site immediately in front of the amino-terminal glycine of Bet v 1. Since Factor Xa hydrolyzes the polypeptide chain adjacent to the recognition site in the carboxy-terminal end, this strategy generated the authentic amino terminus in Bet v 1 after cleavage. Another disadvantage of using the maltose-binding protein fusion construct may lie in incomplete cleavage of fusion protein by incubation with Factor Xa. This problem was overcome by addition of small amounts of the detergent SDS which resulted in almost complete cleavage. This is probably a result of exposure of the Factor Xa cleavage site due to SDS binding to

TABLE 1

Summary of the Yield and Purity after the Major Steps in the Isolation of Recombinant Bet v 1

Protein fraction	Total protein (mg)	Purity (%)
E. coli cell lysate	350	
Amylose affinity-purified fusion protein	50	70
Molecular sieve-purified recombinant Bet v 1	10	>95

Note. Purity of protein fractions was estimated by SDS–polyacrylamide gel electrophoresis.

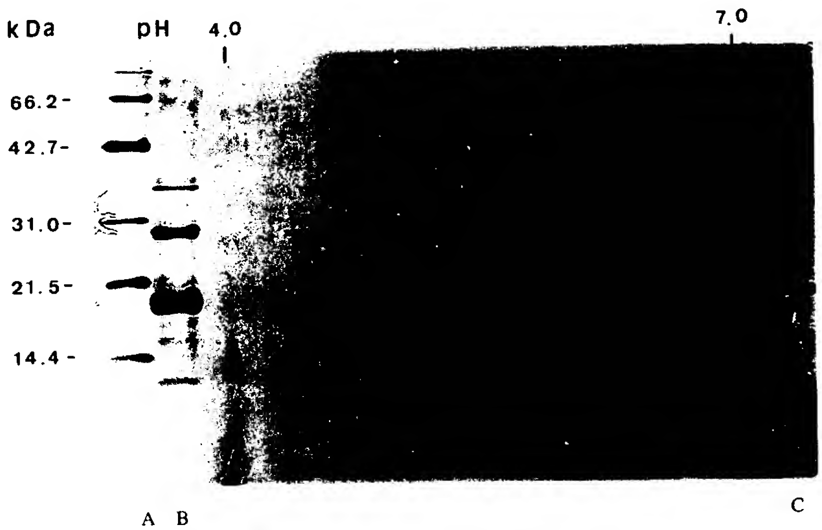

FIG. 3. Silver-stained two-dimensional IEF/SDS–polyacrylamide gel of purified recombinant Bet v 1 in the pH range 4–7. (lane A) Molecular weight markers, (lane B) birch pollen extract, (lane C) purified recombinant Bet v 1.

the fusion construct. Thus, the heterologous bacterial expression system and three-step purification procedure described here result in relatively high yields of recombinant Bet v 1 with the correct N-terminus. Mass spectrometric analysis showed that recombinant Bet v 1 also has the expected molecular mass and amino acid sequence. Characterization of purified recombinant Bet v 1 indicates that it is correctly folded, which was further substantiated by 1-D ^{1}H nuclear magnetic resonance (NMR) spectroscopy, showing spectra typical for a fully folded protein (in preparation).

The isolated recombinant isoallergen gave rise to several protein spots when analyzed by 2-D gel electrophoresis. Each of the protein spots of the recombinant protein coincided with Bet v 1 protein spots present in birch extract. This suggests that both recombinant protein and natural Bet v 1 are chemically modified during 2-D gel electrophoretic analysis, giving rise to charge variants. This is in agreement with the observation that no posttranslational modifications of recombinant Bet v 1 could be detected by mass spectrometry. However, a partial deamination of the amino acids Asn and Gln cannot be excluded since such deamination result in only 1 Da increase in mass, which is below the detection limit of the electrospray mass spectrometry analysis. A partially deamidated peptide would also not be resolved by MALDI mass spectrometry. Thus, the 2-D electrophoretic characterization of recombinant protein suggest that some of the Bet v 1 heterogeneity observed in pollen extract may represent an artifact generated by the analysis system. On the other hand, theoretical p*I* calculations using 10 known Bet v 1 sequences (19) can account for a charge variation spanning over 1.5 pH units, which is exactly as observed for a pollen extract when analyzed by 2-D gel electrophoresis (data not shown). In addition, further heterogeneity probably exist as different Bet v 1 isoallergens can focus under the same protein spot on a 2-D gel.

The immunochemical properties of recombinant Bet v 1 were tested by antibody binding on immunoblots and by its ability to induce *in vitro* T-cell proliferation. The immunoblotting experiments showed that the epitopes defined by monoclonal antibodies raised against natural Bet v 1 also are present on recombinant Bet v 1. Furthermore, the cross-reactivity shown by the monoclonal antibody CB11, raised against the major hornbeam allergen Car b 1, shows that recombinant Bet v 1, natural Bet v 1, and Car b 1 have the epitope defined by this antibody in common. Binding intensity of a birch pollen allergic patient's serum IgE was comparable between recombinant and naturally occurring protein, indicating that the antigenic properties are preserved in the recombinant protein. Since antibody binding is believed to occur predominantly in conformational epitopes, this also suggests that recombinant protein has a three-dimensional structure similar to the natural protein.

A Bet v 1-specific T-cell line established from a birch pollen allergic patient was used to demonstrate the presence of T-cell epitopes in recombinant Bet v 1. The positive control, natural Bet v 1, consists of several Bet v 1 isoallergens and gave a somewhat higher stimulation index compared to recombinant protein. This apparently higher stimulation could be due to the presence of different clones with different Bet v 1 isoallergen specificity in the T-cell line.

In summary, the immunochemical characterization

FIG. 4. Immunoblot of recombinant Bet v 1 using a panel of monoclonal antibodies raised against Bet v 1 purified from birch pollen (lanes 1–7 show reactivity of monoclonal antibodies BV07, BV08, BV09, BV10, BV16, BV18, and BV19, respectively) and monoclonal antibody, CB11, raised against major hornbeam allergen (lane 8) and against monoclonal antibody, CV03 raised against a major allergen from *Chladosporium herbarum* (lane 9). Lanes 10 and 11 show reactivity of rabbit polyclonal antibodies raised against birch pollen extract (v 0991) and naturally occuring purified Bet v 1 (vI 1472), respectively.

FIG. 5. Autoradiogram of immunoblot against recombinant Bet v 1 (left lane) and birch pollen extract (right lane) using a pool of pollen allergic patient's serum IgE.

TABLE 2

	rBet v 1	nBet v 1	PPD
cpm (10 μg/ml)	9414	14493	155
SI	61.3	93.5	1

Note. The table shows the reactivity of a Bet v 1 reactive T-cell line to purified recombinant Bet v 1 (rBet v 1) and to natural Bet v 1 (nBet v 1) at 10 μg/ml. The results are indicated as cpm values and stimulation indices (SI) relative to the background value (PPD). All data are calculated as mean values of triplicate cultures.

presented here shows that recombinant Bet v 1 have B- and T-cell epitopes comparable to those of the naturally occurring protein, in agreement with earlier observations (12,20). This makes the recombinant tree pollen allergen an ideal model system for the study of the effect of specific amino acid substitutions on allergenic epitope structure.

The ability to produce recombinant allergens with intact immunochemical properties and correct amino acid composition and sequence is of major importance for their potential use as therapeutic and diagnostic agents. Although there are several examples of high-expression systems of recombinant pollen allergens as fusion proteins (21–23), a fusion construct does not always show the same immunochemical properties as a purified recombinant or naturally occurring allergen [see, for instance, (21)]. With the improved expression system reported here, studies can now be performed using recombinant Bet v 1 that very closely resemble the natural protein with respect to primary and tertiary structure. Furthermore, the PCR-based cloning strategy is likely to be a generally applicable method for the generation of recombinant proteins with authentic N-terminus from fusion constructs.

ACKNOWLEDGMENTS

We thank Annette Giselsson and Jens Andersen for excellent technical assistance and Henning Løwenstein and Carsten Schou for valuable discussions. This work was supported in part by the Lundbeck Foundation and the Protein Engineering Center under the Danish Biotechnology Program.

Note added in proof. During the preparation of this manuscript the atomic structure of recombinant Bet v 1 has been determined both by X-ray crystallography at 2.0 Å resolution and in solution by NMR spectroscopy (Spangfort *et al.,* submitted for publication).

REFERENCES

1. D'Amato, G., Spieksma, F. T. M., and Bonini, S. (1991) *in* "Allergenic Pollen and Pollinosis in Europe," Blackwell, Oxford.
2. Belin, L. (1972) Separation and characterization of Birch pollen antigens with special reference to the allergenic components. *Int. Arch. Allergy* **42,** 329–342.
3. Viander, M., Fraki, J., Djupsund, B. M., and Laine, S. (1979)

Antigens and allergens in Birch pollen extract. *Allergy* **34,** 289–302.

4. Apold, J., Florvaag, E., and Elsayed, S. (1981) Comparative studies on tree-pollen allergens. *Int. Arch. Allergy Appl. Immunol.* **64,** 439–447.
5. Ipsen, H., and Løwenstein, H. (1983) Isolation and immunochemical characterization of the major allergen of Birch pollen (*Betula verrucosa.) J. Allergy Clin. Immunol.* **72,** 150–159.
6. Ipsen, H., and Hansen, O. C. (1991) The NH_2-terminal amino acid sequence of the immunochemically partial identical major allergens of alder (*Alnus glutinosa*) *Aln g* I, birch (*Betula verrucosa*) *Bet v* I, hornbeam (*Carpinus betulus*) *Car b* I and Oak (*Quercus alba*) *Que a* I pollens. *Mol. Immunol.* **28,** 1279–1288.
7. Ipsen, H., Wihl, J-Å., Nüchel Petersen, B., and Løwenstein, H. (1992) Specificity mapping of patients IgE response towards the tree pollen allergens *Aln g* I, *Bet v* I and *Cor a* I. *Clin. Exp. Allergy* **22,** 391–399.
8. Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O., and Breitenbach, M. (1989) The gene coding for the major birch pollen allergen Bet v I, is highly homologous to a pea disease resistance response gene. *EMBO J.* **8,** 1935–1938.
9. Larsen, J. N., Strøman, P., and Ipsen, H. (1992) PCR based cloning and sequencing of genes encoding the tree pollen major allergen *Car b* 1 from *Carpinus betulus* (hornbeam). *Mol. Immunol.* **29,** 703–711.
10. Breiteneder, H., Ferreira, F., Reikerstorfer, A., Duchene, M., Valenta, R., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Kraft, D., and Scheiner, O. (1992) Complementary DNA cloning and expression in *Escherichia coli* of *Aln g* 1, the major allergen in pollen of alder (*Alnus glutinosa*). *J. Allergy Clin. Immunol.* **90,** 909–917.
11. Breiteneder, H., Ferreira, F., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1993) Four recombinant isoallergens of *Cor a* I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur. J. Biochem.* **212,** 355–362.
12. Ebner, C., Schenk, S., Szepfalusi, Z., Hoffmann, K., Ferreira, F., Willheim, M., Scheiner, O., and Kraft, D. (1993) Multiple T cell specificities for *Bet v* I, the major birch pollen allergen, within single individuals. Studies using specific T cell clones and overlapping peptides. *Eur. J. Immunol.* **23,** 1523–1527.
13. Walter, M. H., Liu, J-W., Grand, C., Lamb, C. J., and Hess, D. (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. *Mol. Gen. Genet.* **222,** 353–360.
14. Bufe, A., Spangfort, M. D., Kahlert, H., Schlaak, M., and Becker, W-H. (1996) The major birch pollen allergen Bet v 1 shows ribonuclease activity. *Planta* **199,** 413–415.
15. Florvaag, E., Holen, E., Vik, H., and Elsayed, S. (1988) Comparative studies on tree pollen allergens. XIV. Characterization of the birch (*Betula verrucosa*) and hazel (*Corylus avellana*) pollen extracts by horizontal 2-D SDS-PAGE combined with electrophoretic transfer and IgE immunoautoradiography. *Ann. Allergy* **61,** 392–400.
16. Jarolim, E., Rumpold, H., Endler, A. T., Ebner, H., Breitenbach, M., Scheiner, O., and Kraft, D. (1989) IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa. Allergy* **44,** 385–395.
17. Løwenstein, H., Sparholt, S. H., Klysner, S. S., Ipsen, H., and Larsen, J. N. (1995) The significance of isoallergenic variations in present and future specific immunotherapy. *Int. Arch. Allergy Immunol.* **107,** 285–289.
18. Larsen, J. N. (1995) Isoallergens, significance in allergen exposure and response. *ACI News* **7,** 141–146.
19. Swoboda, I. (1994) EMBL/GeneBank/DDBJ databases 1994.
20. Larsen, J. N., Casals, A. B., From, N. B., Strøman, P., and Ipsen, H. (1993) Characterization of recombinant *Betv 1,* the major pollen allergen of *Betula verrucosa* (White Birch), produced by fed-batch fermentation. *Int. Arch. Allergy Clin. Immunol.* **102,** 249–258.
21. Ferreira, F. D., Hoffmann-Sommergruber, K., Breiteneder, H., Pettenburger, K., Ebner, C., Sommergruber, W., Steiner, R., Bohle, B., Sperr, W. R., Valent, P., Kunl, A. J., Breitenbach, M., Kraft, D., and Scheiner, O. (1993) Purification and characterization of recombinant *Bet v 1,* the major Birch pollen allergen. *J. Biol. Chem.* **268,** 19574–19580.
22. Sidoli, A., Tamborini, E., Giuntin, I., Levi, S., Volonté, G., Paini, C., De Lalla, C., Siccardi, A. G., Baralle, F. E., Galliani, S., and Arosio, P. (1993) Cloning, expression and immunological characterisation of recombinant *Lolium perenne* allergen *Lol p* II. *J. Biol. Chem.* **268,** 21819–21825.
23. Villalba, M., Batanero, E., Monsalve, R. I., Gonzälez de la Peña, M. A., Lahoz, C., and Rodriguez, R. (1994) Cloning and expression of *Ole e* I, the major allergen from Olive tree pollen. *J. Biol. Chem.* **269,** 15217–15222.
24. Kuo, M-C., Zhu, X-J., Koury, R., Griffith, I. J., Klapper, D. G., Bond, J. F., and Rogers, B. L. (1993) Purification and immunochemical characterization of recombinant and native ragweed allergen *Amb a* II. *Mol. Immunol.* **30,** 1077–1087.
25. Nüchel Petersen, B., Janniche, H., Munch, E. P., Wihl, J-Å., Böwadt, H., Ipsen, H., and Løwenstein, H. (1988) Immunotherapy with partially purified and standardized tree pollen extracts. I. Clinical results from a three-year double-blind study of patients treated with pollen extracts either of birch or combinations of alder, birch and hazel. *Allergy* **43,** 353–362.
26. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *in* "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Press, New York.
27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) "Current Protocols in Molecular Biology," Wiley, New York.
28. Amann, E., and Brosius, J. (1985) 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli. Gene* **40,** 183–190.
29. Hanahan, D., and Meselson, M. (1983) Plasmid screening at high colony density. *Methods Enzymol.* **100,** 333–342.
30. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
31. O'Hehir, R. E., Askonas, B. A., and Lamb, J. R. (1993) Cell culture lymphocyte clones, *in* "Methods of Immunological Analysis," Vol 3 (Albert, W. H. W., and Staines, N. A., Eds.), pp. 120–138, Springer, Heidelberg.
32. Ipsen, H., and Larsen, J. N. (1988) Detection of antigen specific IgE antibodies in sera from allergic patients by SDS-PAGE immunoblotting and crossed radioimmunoelectrophoresis, *in* "Handbook of Immunoblotting of Proteins," Vol II (Bjerrum, O., and Heegard, N. H. H., Eds.), pp. 159–166, CRC Press, Boca Raton.
33. Klysner, S., Welinder, K. G., Løwenstein, H., and Matthiesen, F. (1992) Group V allergens in grass pollens: IV. Similarities in amino acid compositions and NH_2-terminal sequences of the Group V allergens from *Lolium perenne, Poa pratensis* and *Dactylis glomerata. Clin. Exp. Allergy* **22,** 491–497.